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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
C12N 15/49, 15/62, 15/70, 1/21, C07K 14/16, G01N 33/569 // (C12N 1/21, C12R 1:19)	A1	WO 96/12023
		(43) International Publication Date: 25 April 1996 (25.04.96)

(21) International Application Number: PCT/US95/13335

(22) International Filing Date: 11 October 1995 (11.10.95)

(30) Priority Data:
111311 17 October 1994 (17.10.94) IL

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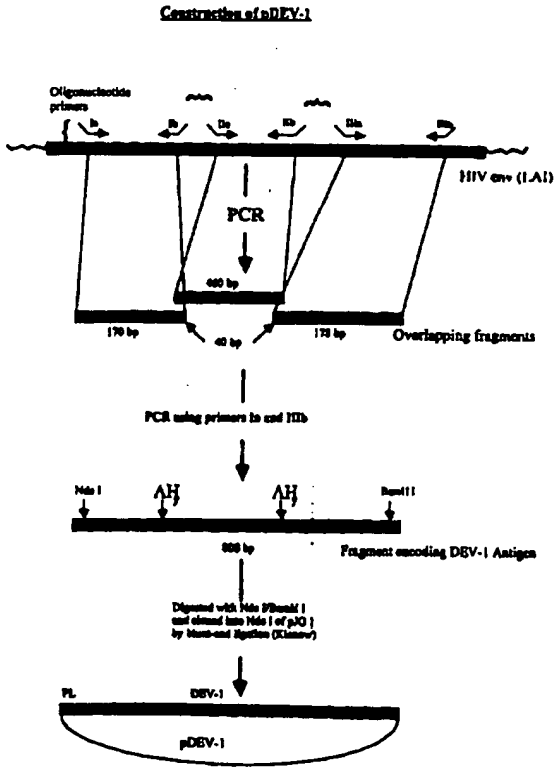
(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A RECOMBINANT PROTEIN DESIGNATED DEV-1, USEFUL IN THE DETECTION OF HIV, DNA SEQUENCE ENCODING THE PROTEIN, AND IMMUNOASSAYS USING THE PROTEIN

(57) Abstract

A purified protein (SEQ ID NO. 1), and biologically active analogs, fragments and derivatives thereof are described, which are useful, *inter alia* as an antigen for the detection of HIV antibodies in human biological samples.



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A RECOMBINANT PROTEIN DESIGNATED DEV-1, USEFUL IN THE DETECTION OF HIV, DNA SEQUENCE ENCODING THE PROTEIN, AND IMMUNOASSAYS USING THE PROTEIN

FIELD OF THE INVENTION

The present invention concerns antigens useful in the diagnosis of the Human Immunodeficiency Virus (HIV). More particularly, the invention relates to a recombinant protein which maximizes sensitive detection of antibodies to HIV and minimizes the danger of false-positive results in immunoassays for HIV, to DNA sequences encoding the protein, and to their use in immunoassays.

BACKGROUND OF THE INVENTION

Human Immunodeficiency Virus (HIV, also HIV, LAV, ARV), a cytopathic lymphotropic retrovirus, is the causative agent of Acquired Immunodeficiency Syndrome (AIDS) in humans. [Gallo, et al., Science, 224:500 (1984); Popovic, et al., Science, 224:497 (1984); Sarngadharan, et al., Science, 224:506 (1984)]. The underlying disease state involves a tropism of HIV for the T4+ lymphocyte subset resulting in a selective depletion of the helper/inducer cells of the immune system, leaving the individual defenseless against a number of opportunistic infections.

Based on recent CDC estimates, over one million persons in the United States are infected with HIV, while WHO estimates fourteen million people, including one million children, are infected worldwide. The WHO projects a cumulative total of about forty million HIV infections in developing countries by the end of the century. Therefore, the development of diagnostics and vaccines to HIV is the subject of intense medical research. The nucleotide sequence of several independent viral isolates of HIV have been determined, [Ratner et al., Nature, 313(6000):227 (1985); Human Retroviruses

and AIDS, Los Alamos National Laboratory, (1991). The viral genome is reported to contain about 10 kilobases which encode four long open reading frames: gag, pol, sor, and env. The env open-reading frame of HIV, which consists of 863 amino acids, has been reported to encode a 160 kd precursor glycoprotein, designated gp160. This precursor glycoprotein is thought to be processed into a 120 kd exterior glycoprotein, designated gp120, and a 41kd transmembrane protein, designated gp41. All three proteins have been found to react with AIDS patient sera. [Barin et al., Science, 228:1094 (1985); Sarngadharan et al., Science, 224:506 (1984). Recombinant proteins derived from the env reading frame and other regions of the HIV genome had been studied as diagnostic and vaccine candidates.

The following references are representative of this still ongoing research. Chang et al., Science, 228:93 (1985) discloses, the expression in *E. coli* of open reading frame gene segments of HIV. Cloned HIV DNA was sheared into approximately 500-base-pair fragments and inserted into an "open reading frame" expression vector. The inserted DNA was expressed in *E. coli* transformants as fusion proteins which were immunoreactive with serum from HIV infected individuals. Reactive fragments were derived from the open reading frame DNA segments corresponding to the gag and pol coding regions and also the open reading frame region env-lor located near the 3' end of the viral genome. Crowl et al., Cell, 41:979 (1985) discloses HIV-1 env gene products synthesized in *E. coli* which are recognized by antibodies present in the sera of AIDS patients. A large segment of the env gene (1800 bp) was inserted into an expression vector. The inserted DNA was expressed in *E. coli* transformants as a recombinant protein containing 611 amino acids which encompassed both the extracellular and the membrane associated regions of the native protein. AIDS patient sera recognized the bacterially synthesized envelope protein in Western blot experiments. Chang et al., Nature, 315:151 (1985) reports the production of a

recombinant 15K peptide encoded by the 3' end of the viral pol gene. The peptide is described as strongly immunoreactive with anti-HIV antibodies present in sera from AIDS patients. Allan et al., Science, 230:810 (1985) discloses a HIV/LAV 27,000 MW protein having a coding origin 3' to the env gene. U.S. Patent 4,520,113, issued to Gallo et al., discloses serological detection of antibodies to HIV in sera of patients with aids and pre-aids conditions. HIV isolated from AIDS patients and transmitted by cocultivation with an HT cell line is detected by antibodies from human sera taken from AIDS patients. The most prominent reactions were reported to be directed to gp41, a 41,000 MW protein constituting the envelope antigen of the HIV virus.

U.S. Patent No. 4,725,669 teaches an assay for detecting infection by human T-cell lymphotropic virus-III, by assaying a biological specimen for the presence of either of two specific proteins.

Indeed, the first generation HIV antibody tests used HIV viral lysate, containing multiple antigens (envelop, gag, pol, etc.). In spite of these antigens, the tests lacked sensitivity and picked up false positive samples. As viral lysates were substituted with recombinants, tests were improved both in sensitivity and specificity. Furthermore, some specific recombinants show superiority as antigens although they are not as long as other recombinants and they contain fewer immunological domains. Their superior performance may be due to folding on the matrix in a way that reflects better their *in vivo* presentation to the immune system.

WO 86/02383 deals with purified expression products of DNA sequences derived from the genome of the LAV virus, and particularly to a glycoprotein having a molecular weight of about 110,000 or antigen of lower molecular weight derived from the

preceding one, which purified product possesses the capacity of being recognized by sera of human origin and containing antibodies against the LAV virus.

Although second generation ELISA tests for HIV showed considerable improvement compared to the first generation, they still tend to produce false positive results. The accepted procedure today is that in addition to the ELISA tests, positive samples are confirmed by Western Blots. In cases when Western Blot is negative or indeterminate, the person is considered HIV negative. Thus, in order to confirm that a person is HIV positive, an ELISA test, that lasts for several hours, and an expensive Western Blot test, that lasts 24 hours, are required.

SUMMARY OF THE INVENTION

It has now been found, and this is an object of the present invention, that it is possible to provide a specific recombinant protein which exhibits substantially improved specificity over prior art antigens, and which therefore, when used as an antigen in immunoassays for HIV infection, leads to results that are substantially more reliable.

It has further been found, and this is another object of the invention, that in addition to the high reliability of the tests employing the protein of the invention, simpler and quicker tests can be provided, which effectively and simply detect HIV infection in human biological samples. Such assays are rapid, simple, and as reliable as the state of the art ELISA in detecting early immune response (Seroconversion). It is therefore an object of the invention to provide a simple and quick test which employs the protein of the invention.

It is still another object of the invention to provide a recombinant DNA sequence which encodes the protein of the invention.

It is yet a further object of the invention to provide an efficient expression system for producing the protein of the invention.

It is also an object of the invention to provide a protein which has improved efficacy, as compared with prior art antigens, in the early detection of HIV seroconvertors.

Other objects and advantages of the invention will become apparent as the description proceeds.

The invention, accordingly, provides a recombinant protein displaying the antigenicity of Human Immunodeficiency Virus (HIV) viral antigens. The protein, designated hereinafter as "DEV-1", comprises the C- terminus of gp120 and most of gp41. Two *E. coli* cytotoxic sections (nuc.acid 7366-7440, and nuc.acid 7855-7929; or AA 517-541 and AA 680-704, of the HIV genome sequence) are deleted from this gp120-gp41 fusion protein, DEV-1, in order to facilitate its expression in *E. coli* cells. DEV-1 starts from HIV-1_{BRU} env amino acid 474, and ends in amino acid 752.

The purified protein was tested for immunoreactivity in ELISA and Western Blots. It is superior to other commercially available antigens in early detection of HIV seroconvertors, and has minimal specificity problems. Furthermore, purified DEV-1 tolerates a wide range of pH and buffer conditions, and can be easily lyophilized and then reconstituted into solution.

The invention thus provides a purified protein, which is the protein of SEQ ID NO. 1, biologically active analogs, fragments and derivatives thereof.

In one aspect, therefore, the invention is directed to a purified protein, which is the protein of SEQ ID NO. 1. In SEQ ID NO. 1, the amino acid residues designated "Xaa" are meant to indicate that these residues are not present in the protein DEV-1, rather, they serve to show those residues which were deleted from the new gp120-gp41 fusion protein. These deleted residues constitute the above-noted *E. coli* cytotoxic sections.

In another aspect, the invention is directed to the use of the protein of SEQ ID NO. 1, as an antigen for the detection of HIV antibodies in human biological samples.

According to a preferred embodiment of the invention, the method of detecting antibodies to HIV in a human biological sample, comprises providing a support to which the protein of SEQ ID NO. 1 is bound, contacting the solid support with the human biological sample, and examining the solid support to determine whether antibodies have been bound to the protein.

The skilled person will easily recognize suitable methods to determine whether antibodies have become bound to the antigen, and any suitable method can be used. In one preferred embodiment of the invention, for instance, the determination of whether antibodies have been bound to the protein is carried out by an ELISA test.

In another preferred embodiment, the determination of whether antibodies have been found is carried out by the use of gold particles conjugated to an antibody-binding protein.

The invention is also directed to a method of producing the protein of SEQ ID NO. 1, comprising providing a vector comprising a DNA sequence essentially the same as SEQ ID NO. 2, and expressing the same in a suitable host cell.

As will be appreciated by the skilled person, there is no limit to the number of possible vectors which can be used to express the protein, and any suitable vector is to be considered equivalent to the vector employed in the examples to follow. According to a preferred embodiment of the invention, however, the vector is plasmid pDEV-1, which has been seen to express the protein in high yield and stability.

Likewise, the protein of the invention can be expressed in a variety of host cells, which will be recognized by the skilled person. According to a preferred embodiment of the invention the host cell is an *E. coli*, e.g., *E. coli* W3110.

Plasmid pDEV-1 is a novel plasmid and as such also forms a part of the present invention. Also encompassed by the invention is *E. coli* W3110, containing plasmid pDEV-1, having inserted into it the recombinant DNA sequence, which is SEQ ID NO. 2, that encodes the protein of the invention.

The present invention also provides a new DNA sequence which encodes the new protein of the invention. The new DNA sequence of the invention is a recombinant DNA sequence encoding the protein DEV-1, biologically active analogs or fragments thereof, said DNA sequence selected from the group consisting of:

(a) a DNA sequence comprising essentially the nucleotide sequence set forth in SEQ ID NO. 2;

(b) DNA sequences capable of hybridization to the DNA sequence of (a) under moderately stringent conditions and which encode biologically active protein DEV-1, analogs or fragments thereof; and

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b), and which encode biologically active protein DEV-1, analogs or fragments thereof.

A preferred embodiment of the above DNA sequence of the invention is a recombinant DNA sequence which is SEQ ID NO. 2.

All the above characteristics and advantages of the invention will be better understood through the following illustrative and non-limitative examples and description.

BRIEF DESCRIPTION OF THE DRAWING

- Fig. 1 is a flow diagram illustrating construction of plasmid pDEV-1 described in Example 1.

- Fig. 2 is a restriction map of plasmid pDEV-1.

DETAILED DESCRIPTION OF THE INVENTION

A recombinant protein having residues from both the gp120 and gp41 domains of HIV has been created which is highly immunoreactive with the sera of HIV infected individuals. The protein comprises the C-terminus of gp 120 and most of gp41. Two *E. coli* cytotoxic sections are deleted from this gp120-gp41 fusion protein to enable expression thereof in *E. coli* cells (nuc.acid 7349-7425, and nuc.acid 7839-7920; or AA 517-541 and AA 680-704 of the HIV genome sequence). The protein starts from HIV-1 env. amino acid 474, and ends at amino acid 752. The specified domains of HIV are known in the art. It is to be understood that the expression "corresponding

to" includes modifications of the specified amino acid sequences which do not adversely affect the antigenic characteristics of the protein of the invention. In addition to the antigenic segment defined above, the protein can contain additional amino acids corresponding to other domains of HIV or other sources, such as a plasmid. One skilled in the art could align the amino acid sequences of peptides from different sources to the scheme in SEQ ID no. 1 to identify the segments therein which correspond to the protein defined herein.

More particularly, the present invention also concerns biologically active analogs, fragments and derivatives of the protein DEV-1. In accordance with the present invention, biologically active analogs are those analogs which have at least a one amino acid change with respect to the amino acid sequence of SEQ ID NO. 1, but which retain essentially all of the antigenic properties, especially the sensitivity to anti-HIV antibodies, of the DEV-1 protein. The at least one amino acid change present in these analogs may be the result of a deletion of one or more amino acid residues, an addition of one or more amino acid residues or a substitution of one or more amino acid residues with different amino acid residues, all with respect to the sequence of SEQ ID NO. 1.

Similarly, biologically active fragments of the invention are those fragments or portions that have been derived from the DEV-1 protein (SEQ ID NO. 1), by substantial deletion of a number of amino acid residues thereof, but which retain essentially all of the antigenic properties, especially the sensitivity to anti-HIV antibodies, of the DEV-1 protein.

The above analogs and fragments of the invention may be readily prepared by any skilled artisan using standard recombinant DNA technology. For example, the plasmid

of the invention, pDEV-1, may be manipulated so as to generate one or more deletions, additions or substitutions of codons in the DEV-1 coding sequence to provide new sequences encoding analogs; or so as to generate substantial deletions in the DEV-1 coding sequence to provide new sequences encoding fragments. Each such analog or fragment may then be readily analyzed for its suitability, i.e., biological activity, by applying the quality tests set forth herein in Examples 2 and 4-7.

Likewise, biologically active derivatives of the invention are those derivatives of the DEV-1 protein, its biologically active analogs and its biologically active fragments, that have been produced by modifying DEV-1, its analogs or fragments, chemically, for example, by acylation, amidation, etc.; or by conjugation with other proteins, such as, for example, enzymes, antibodies, etc., or other molecules such as, for example, lectins, etc.; or by labeling with, for example, radiolabels and fluorescent labels. These derivatives may be prepared by any of the well known methods of the art.

The antigenic sequences of the protein DEV-1 of the invention is highly sensitive to the presence of antibodies against HIV and can serve in their early detection. For this and other reasons, the protein of the invention provides a superior sensitive diagnostic reagent for detecting HIV infection. The sensitivity of the protein permits the early detection of HIV, because it recognizes lower serum titers of antibodies against HIV infection as compared to other known recombinant proteins and peptides. A second advantage is related to the specificity of the protein. The protein is smaller in size compared to prior art recombinant proteins, and thus contains fewer non-critical antigenic domains, thus minimizing the possibility of "false positive" results when employed as a diagnostic reagent to detect HIV infections, as described in more detail below (Table III).

In addition, the protein shows excellent stability over a large range of temperatures, for long periods of time (see Table IV).

In one embodiment, the protein of the invention is used in a diagnostic kit used for detecting antibodies to HIV in a biological sample. The protein can be employed in a method for detecting antibodies to HIV comprising contacting a biological sample with the protein and detecting, by means known in the art, whether specific HIV antibodies are present in the sample. The methods employed in assays of this type are well known to the skilled person and comprise, for instance, assays of the type described in US 5,006,464, the specification of which is incorporated herein by reference.

In a preferred embodiment, the protein comprises an antigenic segment having an amino acid sequence which corresponds to that encoded by the nucleotide sequence of the BglII to BamH1 restriction fragment of HIV env, and most preferably, an amino acid sequence which corresponds to that encoded by the nucleotide sequence of the Bgl II to Hind III restriction fragment of HIV env, which amino acid sequence is shown in SEQ ID NO. 1. The amino acid residues designated "Xaa" in SEQ ID NO. 1 depict the above-noted residues deleted from the gp120-gp41 fusion protein DEV-1 of the invention, i.e., in the DEV-1 protein, these "Xaa" residues are not present.

Methods for digesting, identifying, recovering, and purifying the various nucleotide sequences which encode the protein of the invention are known to those skilled in the art as are methods for ligating the sequences into vectors, transforming host microorganism strains, cloning and recovering products synthesized. Accordingly, the methods will only be described by reference to the specific embodiments of the invention set forth hereinafter.

MATERIALS AND METHODS

Strains and Plasmids

Construction of Host Strain ENV1

The bacterial expression host strain was constructed at Peprotech Inc. by inserting the DNA containing the CI857/Cro regulatory elements of bacteriophage-lambda into *E. coli* strain W3110 according to the methodology outlined in Herrero et. al., Journal of Bacteriology, Nov. 1990, p.6557-6567 and 6568-6572. The specific Protocol is as follows:

1. A DNA fragment (EcoR1-BamH1) consisting of nucleotides 34500 to 39168 of bacteriophage lambda-CI857 was excised from phage DNA and subcloned into the EcoR1-BamH1 sites of pUC18not using the DH5a host strain. The resulting plasmid was designated lambda/pUC18Not.

2. A DNA fragment (not1) containing the CI857/Cro regulatory elements of bacteriophage lambda was excised from lambda RB/pUC18Not and subcloned into the Not1 site pUT/Hg using CC118 (lambda-pir) as the host strain. The resulting strain was designated lambda/pUT/Hg.

3. Plasmid lambdaNot/pUT/Hg was transfected into *E. coli* host strain W3110 using increased resistance to 10 ug/ml $HgCl_2$ as the initial screen for stable insertion of the transposon into the host strain. The use of mercury resistance screen was somewhat tedious and inaccurate due to some natural resistance by the W3110 strain to $HgCl_2$ in the 5-10 ug/ml concentration range. Ultimately, the stable insertion of the lambda CI857/Cro regulatory elements was confirmed by resistance to lambda infection at 30°C and the ability of the resulting strain to regulate, by temperature shift

induction, the expression of genes residing on plasmids and transcribed by the lambda P_L promotor. The resulting strain was designated ENV1.

Source of materials

***E. coli* strain DH5a, lambda CI857 DNA** were purchased from Life Technologies, Inc.

Plasmids pUC18Not, pUT/Hg and *E. coli* host CC118 (lambda-pir) were obtained from Dr. Burt Ensley, Envirogen Inc., Princeton, NJ. who obtained these materials from the laboratory authoring the aforementioned publication (Herrero et. al., Journal of Bacteriology, Nov. 1990, p.6557-6567 and 6568-6572).

E. coli strain W3110 was obtained from Envirogen Inc., Princeton, NJ.

E. coli W3110 was used as the host strains. The plasmid employed was derived from the pEGF/PV, which contains the P_L promotor and the Shine-Dalgarno sequences inserted into the EcoRI-HindIII sites of pUC18. The plasmid, pEGF/PV contains a cDNA segment encoding for the poliovirus 3C protease placed under trp control. The pEXC derivative employed in the Examples maintains the original PvuII site within the pBR322 sequence and thus contains a single BglII site.

Construction of Plasmid pDEV-1

The plasmid pDEV-1 was constructed by combining the NdeI/BamH 1 env fragment obtained by recombinant PCR with the vector pJG1 digested with NdeI. The env fragment was obtained in two steps. First, three different env fragments were obtained by PCR, and second, they were linked in a second PCR to produce an env fragment encoding for the DEV1 HIV env antigen (See Fig. 1).

Restriction endonucleases, T4 DNA ligase, calf intestinal phosphatase, and the Klenow Fragment of DNA polymerase I were obtained from New England Biolabs or Life Technologies, Inc. and used as recommended. *Pfu* DNA polymerase was obtained from Stratagene Cloning Systems and used as recommended. The plasmid pLAI-3 which contains the HIV-$1_{(BRU)}$ provirus was used as template to produce three fragments of the *env* coding sequence by the polymerase chain reaction. Each reaction mix contained 100 pg of pLAI-3, 200mM of dNTP's, 2.5 units of *Pfu* DNA polymerase, 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 60 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 10 mg/ml nuclease-free BSA, and 0.2 mM of each oligonucleotide primer pair (Ia and Ib, IIa and IIb, or IIIa and IIIb). [The primers were designed so that upon amplification 40 bp overlaps would be generated between the 3' end of fragment I and the 5' end of fragment II and between the 3' end of fragment II and the 5' end of fragment III]. The three reaction mixtures were subject to 30 cycles of DNA amplification in a thermal cycler following a single denaturation step of 3 min at 95°C. Each cycle consisted of a denaturation step of 30 sec at 95°C, an annealing step of 30 sec at 30°C, and a polymerization step of 2 min at 72°C. The resulting fragments (I,170 bp; II,460 bp; and III,178 bp) were loaded on an agarose gel and purified using the gene-clean procedure according to the manufacturer's instructions (Bio-101, Inc.). The three fragments were linked in a second PCR in which about 20 ng, 60 ng, and 20 ng of the fragments I, II, and III, respectively were used as templates in a single amplification reaction with the oligonucleotide primers Ia and IIIb. The resulting fragment (808 bp) was digested with the restriction endonucleases NdeI and BamHI for three hours at 37°C in a buffer containing 1 mM Tris-HCl (pH 7.4), 30 mM KCl, 0.1 mM EDTA, 0.1 DTT, 0.0015% Triton X-100 and 5% glycerol. 10 mg of the vector pJG1 were digested with the enzyme NdeI under identical conditions and incubated for an additional hour after adding 2 units of calf intestinal phosphatase.

Both the env fragment and the pJG1 vector were loaded onto an agarose gel, electrophoresed, and purified by the gene-clean procedure as described previously. About 30 ng of each purified fragment were mixed and incubated at room temperature for 30 min with 1 unit of the Klenow fragment of the DNA polymerase I in a reaction mixture containing 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, and 0.2 mM dNTP's. Following inactivation of Klenow by incubation at 70°C for 10 min, 1 unit of the T4 DNA ligase and ATP to a final concentration of 0.2 mM were added to the reaction and incubated at room temperature for 4 hours. A 10 ml aliquot of the ligation reaction was used to transform *E. coli* strain NB210 by the procedure described by Hanahan, J. Mol. Biol., 166:557-580 (1983). The resulting transformants were screened by restriction analysis to determine the presence of the plasmid construction pDEV-1 (Fig. 2).

Analysis of Protein Expression

E. coli strain W3110 containing the plasmid pDEV-1 was grown at 30°C in Luria Broth containing 100 µg/ml of penicillin until an OD_{600} of 0.2-0.4 and then transferred to 42°C for 6 hours. Aliquots were taken at 0 time and 6 hours after induction. The cell pellet from 500 ul of each sample was resuspended in 50 ul of SDS loading buffer, heated at 100°C for 3 min followed by short centrifugation. 5 to 20 ul of sample were analyzed by polyacrylamide gel (12.5%) electrophoresis under denaturing conditions. For quantity control, samples containing different amount of bovine serum albumin were loaded in the same gel. Proteins were visualized by Coomassie blue staining.

Enzyme Linked Immunosorbant Assay (ELISA)

Purified protein was diluted in 60mM carbonate pH 9.6 buffer containing 0.01% azide and 0.00006% SDS to obtain a protein concentration of 100 ng/well. 100 µl aliquots of the protein solution were placed in each well of Immulon II microtiter plates. The

volume used in the binding step set the total volume used for all the rest of the incubations with the exception of the blocking steps. Binding took place at 4°C for about 18 hours. The plates were then washed with PBS + 0.05% Tween 20 (PBS-T). The plates were blocked with 0.5% casein diluent (6.4 g/l NaCL, 0.16 g/l KCL, 0.16 g/l potassium phosphate, 0.916 g/l sodium phosphate, 0.5 ml Tween-20, 3.6 ml of 0.1% Rhodamine-B, 100 ml of 5% casein, total=1.0 L, pH 7.4) for 1 hour at 37°C and were then washed 3X with PBS-T and stored dry at 4°C until they were used.

The plates were reacted with patient sera at a 1:20 dilution in 0.5% casein diluent in the microtiter wells and incubated for 1 hours at 25°C. The plates were then washed with PBS-T, exposed to goat anti human IgG linked to alkaline phosphatase for 1 hour at 25°C and washed with PBS-T. The color reaction was developed by exposure to 72 µg para-nitrophenylphosphate in 100 µl of diethanolamine buffer (1M) with magnesium chloride and 0.02% sodium azide at a pH of 9.8 for 30 minutes at 37°C followed by addition of sodium hydroxide to a concentration of 1 N. The plates were read on a Dynatech MR7000 microtiter plate reader at a wavelength of 405 nm.

The invention will be better understood through the following illustrative and non-limitative Examples.

Example 1

Construction of Plasmid pDEV-1

The plasmid pDEV-1 was constructed by combining the NdeI/BamH 1 env fragment obtained by Polymerase Chain Reaction (PCR) with the vector pJG1 digested with NdeI. The env fragment was obtained in two steps. First, three different env fragments were obtained by PCR, and second, they were linked in a second PCR to produce an env fragment encoding for the DEV1 HIV env antigen (See Fig. 1).

Restriction endonucleases, T4 DNA ligase, calf intestinal phosphatase, and the Klenow Fragment of DNA polymerase I were obtained from New England Biolabs or Life Technologies, Inc. and used as recommended. *Pfu* DNA polymerase was obtained from Stratagene Cloning Systems and used as recommended. The plasmid pLAI-3 which contains the HIV-$1_{(BRU)}$ provirus was used as template to produce three fragments of the *env* coding sequence by the polymerase chain reaction. Each reaction mix contained 100 pg of pLAI-3, 200 µM of dNTP's, 2.5 units of *Pfu* DNA polymerase, 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 60 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 10 µg/ml nuclease-free BSA, and 0.2 µM of each oligonucleotide primer pair (Ia and Ib, IIa and IIb, or IIIa and IIIb). [The primers were designed so that upon amplification 40 bp overlaps would be generated between the 3' end of fragment I and the 5' end of fragment II and between the 3' end of fragment II and the 5' end of fragment III]. The three reaction mixtures were subject to 30 cycles of DNA amplification in a thermal cycler following a single denaturation step of 3 min at 95°C. Each cycle consisted of a denaturation step of 30 sec at 95°C, an annealing step of 30 sec at 30°C, and a polymerization step of 2 min at 72°C. The resulting fragments (I,170 bp; II,460 bp; and III,178 bp) were loaded on an agarose gel and purified using the gene-clean procedure according to the manufacturer's instructions (Bio-101, Inc.). The three fragments were linked in a second PCR in which about 20 ng, 60 ng, and 20 ng of the fragments I, II, and III respectively were used as templates in a single amplification reaction with the oligonucleotide primers Ia and IIIb. The resulting fragment (808 bp) was digested with the restriction endonucleases NdeI and BamHI for three hours at 37°C in a buffer containing 1 mM Tris-HCl (pH 7.4), 30 mM KCl, 0.1 mM EDTA, 0.1 DTT, 0.0015% Triton X-100 and 5% glycerol. 10 µg of the vector pJG1 were digested with the enzyme NdeI under identical conditions and incubated for an additional hour after adding 2 units of calf intestinal phosphatase. Both the env fragment and the pJG1 vector were loaded onto an agarose gel,

electrophoresed, and purified by the gene-clean procedure as described previously. About 30 ng of each purified fragment were mixed and incubated at room temperature for 30 min with 1 unit of the Klenow fragment of the DNA polymerase I in a reaction mixture containing 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, and 0.2 mM dNTP's. Following inactivation of Klenow by incubation at 70°C for 10 min, 1 unit of the T4 DNA ligase and ATP to a final concentration of 0.2 mM were added to the reaction and incubated at room temperature for 4 hours. A 10 µl aliquot of the ligation reaction was used to transform *E. coli* strain W3110 by the procedure described by Hanahan, J. Mol. Biol., 166:557-580 (1983). The resulting transformants were screened by restriction analysis to determine the presence of the plasmid construction pDEV-1.

The env fragment inserted into the pJG1 vector was sequenced by conventional procedures and the predicted protein product encoded by the env fragment of the resulting plasmid (DEV-1) was shown to contain 233 amino acids which correspond to 38 amino acids of the C-terminal of the gp120 domain and 241 amino acids of the N-terminal of the gp41 domain, but from which gp120-gp41 fusion protein there has been deleted two sections observed to be cytotoxic to *E. coli* cells (results not shown). The sequence of the env fragment encoding this gp120-gp-41 fusion protein, herein designated DEV-1, is set forth in SEQ ID NO. 2, and the predicted or deduced amino acid sequence of the DEV-1 protein is set forth in SEQ ID NO. 1, in which the residues "Xaa" depict those which are not present in the protein, but which were deleted therefrom because of their cytotoxic effects on *E. coli* cells.

The *E. coli* strain W3110, containing plasmid pDEV-1 was deposited on June 29, 1994 with, and made permanent part of the collection of, the ARS Patent Culture Collection, Peoria Illinois, U.S.A., under Accession Number NRRL 21291.

Example 2

Reactivity of DEV-1 with Virus Specific Antibodies

Monoclonal antibodies raised to gp41 viral protein were found to react specifically with DEV-1 when tested in immunoblot and ELISA according to methods described previously. The monoclonal antibodies raised to gp41 viral protein employed in this example are commercially available from American Bio-Technology, Inc. (Catalog No. 1577). These results confirm that the viral antigenic sites resides within DEV-1.

Example 3

Purification of Recombinant Protein DEV-1

The protein DEV-1 was purified from the *E. coli* cells containing the vector pDEV-1 described in Example 1 according to the following procedure. The *E. coli* were grown in a rich medium (Difco Lbs, 10-5-5 media, pH 7.2 with Amp. to 100ug/ml) at 30°C and 250 rpm. 10 mls were transferred into 2 liter flask and were grown to 06-0.8 OD then transferred into 42°C incubator. The resulting culture was centrifuged. The pellet was resuspended in dilute buffer and sonicated. After centrifugation, the lysed cell pellet was extracted with 8M urea, 25 mM BICINE pH8.5, 10mM DTT, 5mM EDTA, 0.1% Tween-20.

40-50 grams of inclusion bodies were mixed with 500 mls of the Extract buffer and shaken slowly O/N. The sample was then centrifuged at room temp, 20,000 rpm, 12 min., and the supernatant was collected. (The pellet was re-suspended and a polyacrylamide gel electrophoresis was run to determine a complete extraction).

Q-sepharose (Pharmacia) was pre-equilibrated with extract buffer. The extract was passed through 200 ml of Q-sepharose column, collecting the flow through. PAGE

was run to determine DEV-1 concentration. 150-200 mls of S-sepharose(Pharmacia) was pre-equilibrated with S-sepharose binding buffer (8M Urea, 25 mM BICINE pH 8.5, 10 mM DTT,5 mM EDTA, 0.1% Tween 20).

Q-sepharose flow through was added to the S-sepharose while stirring slowly for 30 min. S-sepharose was then loaded with the flow through onto the column. S-sepharose was then washed with the S-sepharose binding buffer until base line reached (OD 280 = 0.00). DEV-1 was eluted by a linear gradient of 200 ml S-sepharose binding buffer to 200 ml of S-sepharose elute buffer (8M Urea, 25 mM BICINE pH 8.5, 10 mM DTT, 0.4 M NaCl). OD 280 samples were grouped (about 4-6 groups) and then tested by PAGE. The appropriate samples were pooled and tested again by PAGE.

1% SDS was added to the S-sepharose pool. The mixture was then heated to 37°C for 30 min. then diluted 1 to 10 with HA buffer (10 mM NaH_2PO_4, pH 6.0, 10 mM DTT, 0.1% SDS). Pre-equilibrated 150-200 mls of Hydroxylapatite with HA buffer were loaded with the diluted S-sepharose pool. The column was washed until the base line reached (OD 280 = 0.00). DEV-1 was eluted with HA Elute #1 (310 mM NaH2PO4, pH 6.0, 1 mM DTT, 0.1% SDS). When the base line was reached again (OD 280 = 0.00), DEV-1 was eluted with HA Elute #2 (400 mM NaH2PO4, pH 6.0, 1 mM DTT, 0.1% SDS). The eluted DEV-1 was concentrated to about 20-30 mls, and 0.9% SDS and 9 mM DTT were added. S-300 was pre-equilibrate with S-300 sizing buffer (8M Urea, 25 mM BICINE pH 8.5, 5 mM b-Me 5 mM EDTA, 0.1% SDS).

DEV-1 was loaded and fractions were collected. PAGE was then run to determine purity. Finally, DEV-1 was concentrated and dialyzed to the final buffer.

Example 4

Immunoreactivity of DEV-1

Purified DEV-1 was employed to detect antibodies to HIV in AIDS patient sera. The reactivity of HIV positive sera at a dilution of 1:500 gave results above background at a concentration of antigen of 100ng/well for the peptide. HIV positive sera and a number of negative sera were tested. The results of testing 328 negative and 53 positive samples with the assay described above are shown in Table I.

Table I

Frequency distribution of seropositive and seronegative Individuals with DEV-1 ELISA

ELISA Assay	Status	Number of individuals in each absorbance range					
		0-0.2	0.2-0.4	0.4-0.6	0.6-0.8	0.8-1.0	>1.0
	Positive	0	0		0	0	53
	Negative	319	9	0	0	0	0

The seropositive samples used in this study were diagnosed AIDS patients. These were confirmed by other ELISA assays as well as by immunoblot. Samples were run at a dilution of 1:20.

Example 5

A kit containing DEV-1 was manufactured, essentially as described in U.S. Patent No. 5,006,464, and employed to determine its ability to detect early seropositive HIV infected individuals. Seroconversion panels were obtained from Boston Biomedica, Inc. The ability to detect HIV antibodies was compared to a state-of-the-art ELISA (Abbott) and to western blot results. Table II shows that the DEV-1 kit performs as well as the ELISA and Western Blot in detection of infection. Note that the time

required to obtain a result with the Dev-1 Kit is minutes, compared to hours for the ELISA and even days for Western Blot.

Table IIA

1. Anti-HIV-1 seroconversion Panel D

Sample	Abbott 1/2	Western Blot	Dev-1 kit
PRB 904-1	negative	negative	negative
PRB 904-2	negative	negative	negative
PRB 904-3	negative	negative	negative
PRB 904-4	positive s/co 12.0	positive 18,24,41,55,65,120,160	positive
PRB-904-5	positive s/co 12.2	positive 18,24,41,51,55,65,120,160	positive

s/co=seroconversion

According to BBI's information, similar results were obtained in tests performed by kits manufactured by seven other companies. The s/co values were low in all these assays, ranging between 2.2 and 7.3.

Table IIB

2. Anti HIV-1 Seroconversion Panel J.

Sample	Abbott 1/2	Western Blot	Dev-1 kit
PRB 910-1	negative	negative	negative
PRB 910-2	negative	negative	negative
PRB 910-3	positive s/co 10.4	positive 24,51,65,160	positive
PRB 910-4	positive s/co 7.4	positive 24,51,65,160	positive
PRB 910-5	positive s/co 7.6	positive 24,51,65,160	positive
PRB 910-6	positive s/co 7.1	positive 24,51,65,160	positive
PRB 910-7	positive s/co 7.8	positive 24,51,65,160	positive

s/co=seroconversion

According to BBI's information, similar results were obtained in tests performed by kits manufactured by seven other companies. The s/co values ranging between 1.8 and 6.2 were low in all these assays.

Example 6

The Dev-1 kit was tested in performance tests in many different parts of the world, with samples from widely different populations. In all studies the kit has performed at least as well as the much more complex ELISA-based assays and yet is much easier and faster to perform. In addition, the kit does not produce false positive results known to occur in ELISA assays and is extremely well correlated with the Western Blot.

The kit was compared to a leading ELISA test (Abbott) and to the confirmatory Western Blot method (Table III) with the following types of samples:

- samples from known HIV- positive patients. (100 patients)
- samples from known HIV- negative patients. (150 patients)
-samples that are indeterminate by Western Blot. (50 patients)

Table III

Evaluation of Dev-1 Kit

Number of Samples	Abbott 1/2	Dev-1 Kit Rapid SeroTest™	Western Blot
100	P	P	P
150	N	N	N
33	P	N	indeterminate
17	N	N	indeterminate

Specificity: **100%**
Sensitivity: **100%**
compared to Western Blot

Note that the Dev-1 Kit performed better than the ELISA in cases of indeterminate western blot results. Out of 50 such sera that are not considered HIV positive, the ELISA falsely recognized 33 sera as positive, while the Dev-1 Kit showed a negative result for all 50 cases.

Example 7

Stability Tests

The Dev-1 Kit was stored at three temperatures and then tested according to the following schedule:

Table IV

TESTING DATES

	WEEKS					MONTHS										
STORAGE TEMP.	0	1	2	3	4	2	3	4	5	6	7	8	9	10	11	12
4	X				X	X	X	X	X	X	X	X	X	X	X	X
25	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
45	X	X	X	X	X	X										

The devices were tested at each date using a seven member panel derived from the rabbit anti-DEV-1 and the anti-DEV-2 mAb. The results show excellent stability as measured by the sensitivity to the panel . The results indicate that the device is stable for 12 months, at room temperature, and for two months under very harsh conditions.

As will be appreciated by the skilled person, the test carried out according to the invention achieves two important goals: it is as accurate as the western blotting test, but is very quick. Accordingly, each single test becomes very inexpensive, while avoiding false positive results. Thus, the invention provides an important solution to a

problem with which the art has been struggling daily, viz. it provides an accurate, quick and inexpensive means of testing for HIV.

While the description given above has been provided for the purpose of illustration, it is not intended to limit the scope of the invention in any way. The skilled person will be able to carry out many modifications, to prepare longer proteins including the protein of the invention, to prepare shorter proteins including the essential parts of the protein of the invention, to manufacture different kits using the protein, and to use them in different tests, all without exceeding the scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: DEVARON, INC.
(B) STREET: FRESH PONDS CORPORATE VILLAGE, 2235 ROUTE 130
(C) CITY: DAYTON, NEW JERSEY
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 08810
(G) TELEPHONE: 908 2740080
(H) TELEFAX: 908 2740067

(ii) TITLE OF INVENTION: A RECOMBINANT PROTEIN USEFUL IN THE DETECTION OF HIV, DNA SEQUENCES ENCODING THE PROTEIN, AND IMMUNOASSAYS USING THE PROTEIN.

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 282 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Met Ser Ile Asp Met Arg Asp Asn Trp Arg Ser Glu Leu Tyr Lys Tyr
1 5 10 15

Lys Val Val Lys Ile Glu Pro Leu Gly Val Ala Pro Thr Lys Ala Lys
20 25 30

Arg Arg Val Val Gln Arg Glu Lys Arg Ala Val Gly Ile Gly Xaa Xaa
35 40 45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
50 55 60

Xaa Xaa Xaa Xaa Xaa Xaa Ser Arg Gln Leu Leu Ser Gly Ile Val Gln
65 70 75 80

Gln Gln Asn Asn Leu Leu Arg Ala Ile Glu Ala Gln Gln His Leu Leu
85 90 95

Gln Leu Thr Val Ala Gly Ile Lys Gln Leu Gln Ala Arg Ile Leu Ala
100 105 110

Val Glu Arg Tyr Leu Lys Asp Gln Gln Leu Leu Gly Ile Trp Gly Cys
115 120 125

Ser Gly Lys Leu Ile Cys Thr Thr Ala Val Pro Trp Asn Ala Ser Trp
130 135 140

Ser Asn Lys Ser Leu Glu Gln Ile Trp Asn Asn Met Thr Trp Met Glu
145 150 155 160

Trp Asp Arg Glu Ile Asn Asn Tyr Thr Ser Leu Ile His Ser Leu Ile
165 170 175

Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln Glu Leu Leu Glu
180 185 190

Leu Asp Lys Trp Ala Ser Leu Trp Asn Trp Phe Asn Ile Thr Asn Trp
195 200 205

Leu Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
210 215 220

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Val Asn Arg Val Arg Gln
225 230 235 240

Gly Tyr Ser Pro Leu Ser Phe Gln Thr His Leu Pro Ile Pro Arg Gly
245 250 255

Pro Asp Arg Pro Glu Gly Ile Glu Glu Glu Gly Gly Glu Arg Asp Arg
260 265 270

Asp Arg Ser Ile Arg Leu Val Asn Gly Ser
275 280

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 699 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATGAGTATTG ATATGAGGGA CAATTGGAGA AGTGAATTAT ATAAATATAA AGTAGTAAAA 60

ATTGAACCAT TAGGAGTAGC ACCCACCAAG GCAAAGAGAA GAGTGGTGCA GAGAGAAAAA 120

AGAGCAGTGG GAATAGGAAG TAGACAATTA TTGTCTGGTA TAGTGCAGCA GCAGAACAAT 180

TTGCTGAGGG CTATTGAGGC GCAACAGCAT CTGTTGCAAC TCACAGTCTG GGGCATCAAG 240

CAGCTCCAGG CAAGAATCCT GGCTGTGGAA AGATACCTAA AGGATCAACA GCTCCTGGGG 300

ATTTGGGGTT GCTCTGGAAA ACTCATTTGC ACCACTGCTG TGCCTTGGAA TGCTAGTTGG 360

AGTAATAAAT CTCTGGAACA GATTTGGAAT AACATGACCT GGATGGAGTG GGACAGAGAA 420

ATTAACAATT ACACAAGCTT AATACATTCC TTAATTGAAG AATCGCAAAA CCAGCAAGAA 480

AAGAATGAAC AAGAATTATT GGAATTAGAT AAATGGGCAA GTTTGTGGAA TTGGTTTAAC 540

ATAACAAATT GGCTGGTGAA TAGAGTTAGG CAGGGATATT CACCATTATC GTTTCAGACC 600

CACCTCCCAA CCCCGAGGGG ACCCGACAGG CCCGAAGGAA TAGAAGAAGA AGGTGGAGAG 660

AGAGACAGAG ACAGATCCAT TCGATTAGTG AACGGATCG 699

CLAIMS:

1. A purified protein, which is the protein of SEQ ID NO. 1, biologically active analogs, fragments and derivatives thereof.

2. Use of the protein of claim 1, as an antigen for the detection of HIV antibodies in human biological samples.

3. A method of detecting antibodies to HIV in a human biological sample, comprising providing a support to which the protein of SEQ ID NO. 1 is bound, contacting the said solid support with the said human biological sample, and examining the said solid support to determine whether antibodies are bound to the said protein.

4. A method according to claim 3, wherein the determination of whether antibodies are bound to the protein includes the use of contrast agents and/or color materials conjugated to an antibody binding protein, or an ELISA test.

5. A method according to claim 4, wherein the contrast agent/coloring material includes gold particles.

6. A method of producing the protein of SEQ ID NO. 1, comprising providing a vector comprising a DNA sequence essentially the same as SEQ ID NO. 2, and expressing the same in a suitable host cell.

7. A method according to claim 6, wherein the vector is plasmid pDEV-1.

8. A method according to claim 7, wherein the host cell is an *E. coli*.

9. A method according to claim 8, wherein the host cell is *E. coli* W3110.

10. Plasmid pDEV-1.

11. *E. coli* W3110, containing plasmid pDEV-1.

12. A recombinant DNA sequence encoding the protein DEV-1, biologically active analogs or fragments thereof, said DNA sequence selected from the group consisting of:

(a) a DNA sequence comprising essentially the nucleotide sequence set forth in SEQ ID NO. 2;

(b) DNA sequences capable of hybridization to the DNA sequence of (a) under moderately stringent conditions and which encode biologically active protein DEV-1, analogs or fragments thereof; and

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b), and which encode biologically active protein DEV-1, analogs or fragments thereof.

13. A recombinant DNA sequence, according to claim 12, which is SEQ ID NO. 2.

Construction of pDEV-1

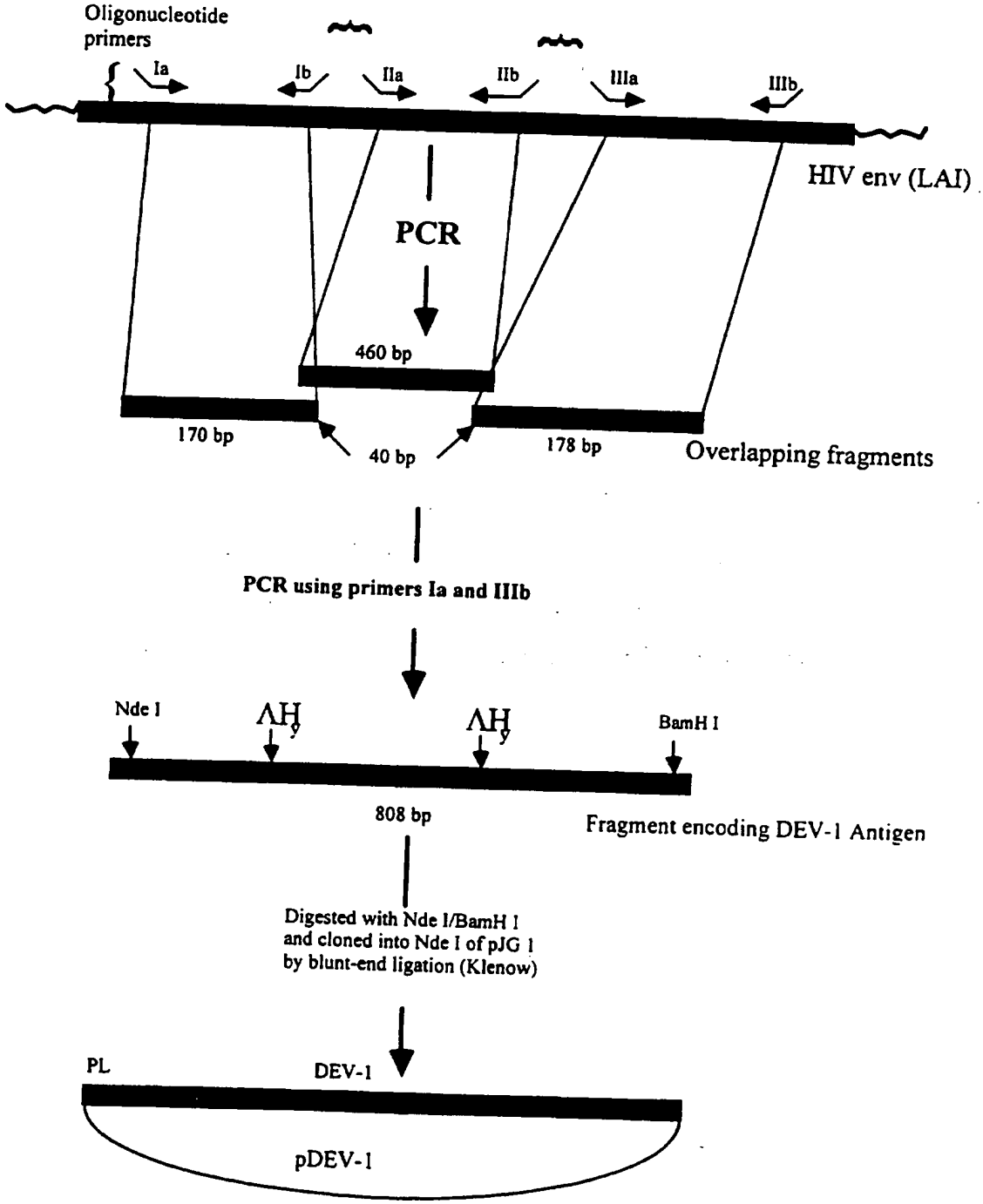

Fig. 1

Restriction map of pDEV-1

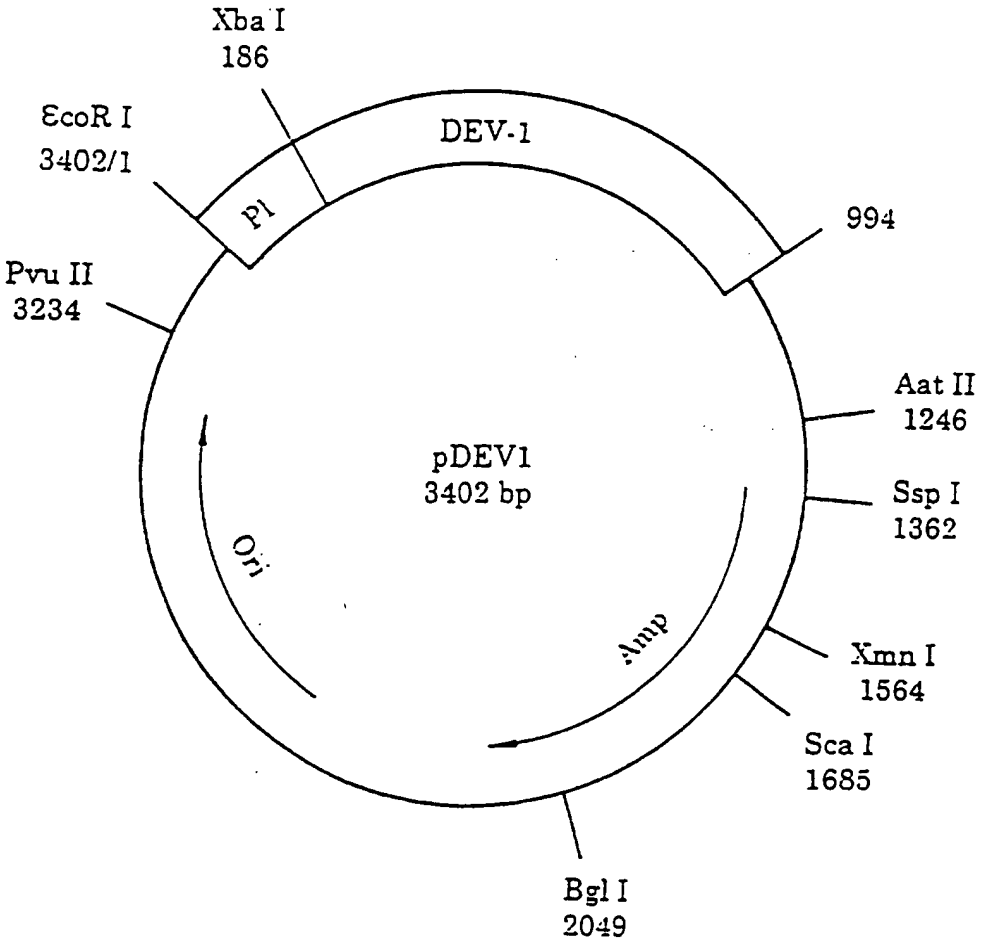

Fig. 2

INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/US 95/13335

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/49 C12N15/62 C12N15/70 C12N1/21 C07K14/16
G01N33/569 //(C12N1/21,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 00594 (ABBOTT LABORATORIES) 6 January 1994 see the whole document	1-4

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
4 March 1996	25.03.96

Name and mailing address of the ISA	Authorized officer
European Patent ffice, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/13335

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9400594	06-01-94	US-A-	5322769	21-06-94
		AU-B-	4643693	24-01-94
		CA-A-	2136765	06-01-94
		EP-A-	0647277	12-04-95
		JP-T-	7508411	21-09-95